Confirming the Presence of Drugs of Abuse in Samples Using an LC/Ion Trap Mass Spectrometer and a Nearly 400 Drugs Compound Library Containing MS^2 and MS^3 Spectra

Application

Forensics/Toxicology

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Abstract

The presence of drugs of abuse is confirmed in samples analyzed in three independent labs using a library of almost 400 drugs of abuse and pharmaceutical compounds containing MS/MS (MS^2) and MS/MS/MS (MS^3) spectra. By ramping the collision energy during the fragmentation stage of analysis, reproducible product ion spectra are generated for the library. By doing this in an ion trap, the spectra represent specific pathway steps of fragmentation. In order to identify the compounds in real samples, the generation of product ion spectra in those samples is also carried out using ramped collision energy.

The process of confirmation includes the generating of compound spectra in the sample data, followed by NIST-based scoring of Fit, Reverse Fit, and Purity matching of library spectra. Searching of the MS spectra is not included because matrix interferences can result in poor scoring. Besides, the precursor ions seen in the MS scan are recorded in the MS^2 and MS^3 level scans.

Finally, an automated procedure for processing the data to generate compound spectra and search them, followed by printing, is presented. The printing of results is limited to just those compounds that are identified, but the overall scoring for each compound takes into account that multiple spectra at multiple levels of fragmentation may match spectra of different compounds in the library. It is this combination of spectral matching at the MS^2 and MS^3 levels that makes searching ion trap libraries not only a unique tool for identification, but also more reliable if a reproducible technique for generating product ions, such as a ramped collision energy, is employed.

In this work, a library has been generated by the Riverview Hospital using an Agilent LC/MSD Trap XCT mass spectrometer, operated with a ramped collision energy of 30% to 200% of the set collision energy, which in this case was 1 V. The ramped voltage of 0.3 to 2.0 V was implemented for 40 msec to generate MS^2 spectra from an isolated precursor ion. The process was repeated on an isolated product ion to produce MS^3 spectra where possible.

This library is used to confirm the presence of certain drugs in a sample analyzed by Riverview Hospital. The library is then used to identify samples at St. Olav's Hospital and Athens Doping Center that they analyzed by their respective Agilent LC/MSD Trap systems. In each case, spectral matches are shown to demonstrate the reproducible nature of generating product ion spectra on the Agilent LC/MSD Trap mass spectrometer. Implementation of automated analysis is also demonstrated.

Introduction

The ion trap mass spectrometer is known for having the most sensitivity for full-scan MS^2 acquisition. Fragment ion spectra are considered "fingerprints" of molecules because they contain product ion masses that correspond to smaller pieces of the overall structure of the molecule. Although other mass spectrometers like the QQQ and the QTOF are able to produce product ions, the process is not exactly the same, which leads to a different characteristic of fragment ion spectra. In the case of a QQQ or QTOF instrument, both of which produce true MS/MS spectra, the product ion spectra typically represent not only primary fragmentation of the precursor ion, but also secondary, tertiary, and so on. That is, after the precursor ion is fragmented while still near the entrance to the collision cell, the produced fragment ions may undergo further fragmentation before exiting toward the third quadrupole.

As a result, the final MS/MS spectrum produced from either the QQQ or QTOF will include product ions that may have come from the precursor or from other product ions. Therefore, fragmentation pathways are unknown. On the other hand, an ion trap is much more selective in fragmenting precursor ions. Following the isolation process, energy is added into the system corresponding to the frequency of oscillation of the precursor ion. The frequency of oscillation is directly related to the mass of the precursor. The precursor ion then absorbs the additional energy and fragments. This is known as resonance excitation.

However, since the produced fragment ions have different masses from the precursor, and thus have different frequencies of oscillation, they do not absorb the additional energy so they are not further fragmented. As a result, the product ions seen in the MS/MS spectrum had to come directly from the precursor ion. Further isolation of a product ion, followed by fragmentation, produces product ions that also lie on a direct pathway back to the precursor ion. Consequently, the ion trap is considered to be a softer, but more specific, MS/MS mass spectrometer than the QQQ or QTOF. Furthermore, the product ion spectra are typically cleaner in the ion trap than in the other instruments. The additional step of forming MS^3 product ions increases the specificity of the precursor ion structure, making the ion trap a unique instrument for structural elucidation.

The caveats of trying to generate MS^3 spectra is that there may not be enough precursor ion left at the MS^2 level to generate an appreciable signal at the MS^3 level or the MS^2 fragment ion may be too stable for further fragmentation. In either case, MS^3 spectra are not available.

Along with increased specificity, there are a couple more benefits to acquiring MS^3 spectra. One is that many compounds may only fragment to form water-loss ions $(M + H - H_2O)^+$, which are not very informative. A further generation of fragment ions in the MS^3 spectra may increase information content drastically. Another benefit of generating MS^3 spectra is that co-eluting, isobaric interferences may contribute product ions at the MS^2 level, which are filtered out at the MS^3 level. Therefore, MS^3 spectra are typically very clean.

The Agilent LC/MSD Trap has an additional feature that enables it to outperform its competitors by generating reproducible product ion spectra. This reproducibility from instrument to instrument is based primarily on the technique known as SmartFrag, which involves a ramping of the fragmentation energy (in volts) during the sequence of fragmentation in the ion trap. The default range of 30% to 200%, with a set value of 1 V, is typically used because 0.3 V (30%) is generally not enough to fragment most precursor ions, and 2.0 V (200%) is too much. However, using a range with such extremes means that the onset of fragmentation for any particular molecular ion will almost always be within the range.

In general, the onset of fragmentation, or the lowest voltage to produce fragments, results in the production of higher mass fragment ions. By ramping to higher voltages, smaller and smaller fragment ions are produced. The overall result is to produce fragment ions across a wide product ion mass range, which is very useful for library identification. (See Figure 1 for an example of how this works.) Furthermore, such a wide range takes into account subtle differences between ion traps in terms of geometry and buffer gas pressure in the ion trap mass analyzer. This results in the ability to generate a library of spectra on one instrument and use it for searching on other instruments.

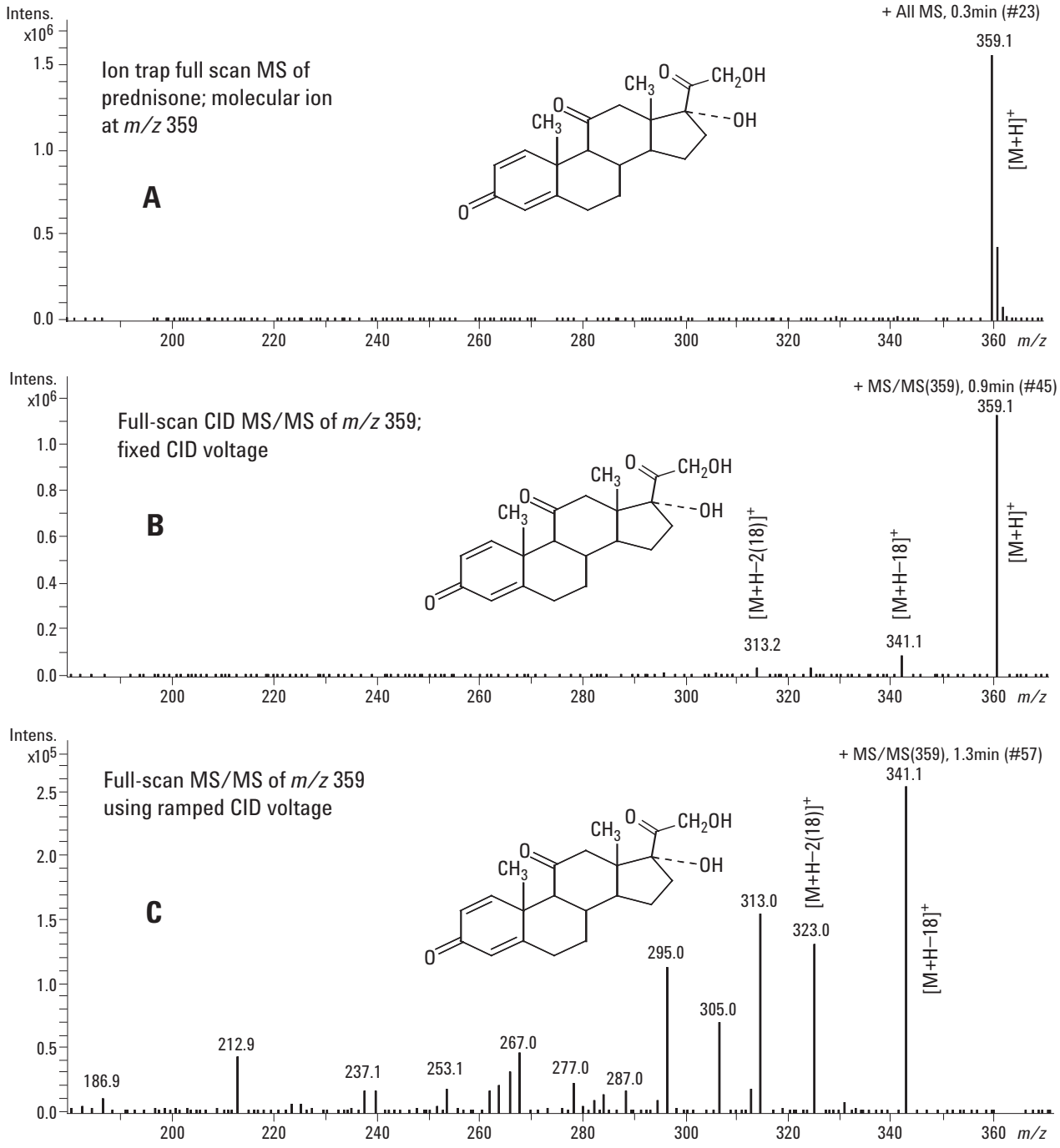

Figure 1. Illustration of using SmartFrag to generate product ion spectra of prednisone across a wide mass range. Spectrum A represents no fragmentation at all. Spectrum B represents a typical fragmentation voltage of 1 V, which produces very little fragmentation. Spectrum C is the result of implementing SmartFrag, or a ramp of 0.3 to 2.0 V, to produce fragment ions across a wide mass range. Note that no precursor ion intensity remains in Spectrum C.

Table 1. List of 386 Drug Compounds in "Drugs_lib" Library

7-Acetamidoclonazepam
7-Acetamidonitrazepam
8-Chlorotheophylline
Acebutolol
Acetamidoclonazepam
Acetaminophen
Acetazolamide
Acetohexamide
Acetylaminoglutethimide
Acetylcodeine
Acetylprocainamide
Acyclovir
Alfentanil
Alphaprodine
Alprazolam
Amantadine
Aminoclonazepam
Aminoflunitrazepam
Aminoglutethimide
Amiodarone
Amisulpiride
Amitriptyline
Amlodipine
Amobarbital
Amoxapine
Amphetamine
Anhydroecognine
Anhydroecognine methyl ester
Anileridine
Aprobarbital
Atenolol
Atomoxetine
Atropine
Azatadine
AZT
Baclofen
Bamethane
Benzoylecgonine
Benzoylecognine
Benztropine
Bosentan
Bromacil
Bromazepam
Bromodiolone
Brompheniramine
Bupivacaine
Buprenorphine
Bupropion
Butabarbital
Butalbital
Butorphanol
Butriptyline
Caffeine
Captopril
Carbamazepine
Carbamazepine epoxide
Carboxytetrahydrocannabinol
Carbromal
Carisoprodol
Celecoxib
Cephalexin
Cetirizine
Chlordiazepoxide
Chloroquine
Chlorpheniramine
Chlorpromazine
Chlorpropamide
Chlorprothixene
Cimetadine
Cimetidine
Citalopram
Clenbuterol
Clobazam
Clomipramine
Clonazepam
Clonidine
Clozapine
Cocaethylene
Cocaine
Codeine
Colchicine
Corticosterone
Cortisone
Cotinine
Cyclizine
Cyclobenzaprine
Cyclophosphamide
Cyproheptadine
Debrisoquine
Demoxepam
Desalkyldisopyramide
Desalkylflurazepam
Desipramine
Desmethylchlordiazepoxide
Desmethylclobazam
Desmethylclomipramine
Desmethyldiazepam
Desmethyldoxepin
Desmethylflunitrazepam
Desmethylmaprotiline
Desmethylmethsuximide
Desmethylnaproxen
Desmethyltrimipramine
Dexamethasone
Dextromethorphan
Diazepam
Diazoxide
Diclofenac
Diethylpropion
Dihydroergotamine
Diltiazem
Dimethoate
Dinoseb
Diphenhydramine
Diphenoxylate
Diphenylpyraline
Dipyridamole
Disopyramide
Donepezil
Dopamine
Doxepin
Doxylamine
Droperidol
Ecognine
EDDP
Emetine
Enalapril
Encainide
Ephedrine
Eprosartan
Erythromicin
Ethylmorphine
Fenazepam
Fenfluramine
Fenoprofen
Fenoterol
Fentanyl
Flecainide
Fluconazole
Fludrocortisone acetate
Flunarizine
Flunitrazepam
Fluoxetine
Flupenthixol
Fluphenazine
Flurazepam
Fluvoxamine
Furosemide
Gabapentin
Gentamicin
Gliclazide
Glipizide
Glyburide
Glyphosate
Guaifenesin
Haloperidol
Heroin
Hexobarbital
Hydrochlorothiazide
Hydrocodone
Hydrocortisone
Hydromorphone
Hydroxyalprazolam
Hydroxyamoxapine
Hydroxybupropion
Hydroxychloroquine
Hydroxydesipramine
Hydroxyhaloperidol
Hydroxyrisperidone
Hydroxytriazolam
Hydroxyzine
Ibuprofen
Imipramine
Indomethacin
Isoniazid
Isoproterenol
Itraconazole
Ketamine
Ketazolam
Ketoconazole
Ketoprofen
Ketorolac
Labetolol
Lamotrigine
Leviteracetam
Levodopa
Levorphanol
Lidocaine
Loperamide
Lorazepam
Loxapine
LSD
Maprotiline
Mazindol
MDA

Table 1. List of 386 Drug Compounds in "Drugs_lib" Library (continued)

MDA-NH_3 loss
MDEA
MDMA
Mecoprop
Mefenamic acid
Melatonin
Meperidine
Mephenytoin
Mephobarbital
Mepivacaine
Meprobamate
Mescaline
Metanephrine
Metformin
Methadone
Methamphetamine
Methapyriline
Methaqualone
Methocarbamol
Methotrexate
Methotrimeprazine
Methsuximide
Methylclonazepam
Methyldopa
Methylecognine
Methylphenidate
Methylprednisolone
Methyprylon
Metoclopramide
Metoprolol
Metronidazole
Mexiletine
Midazolam
Mirtazapine
Moclobemide
Monoacetylmorphine
Morphine
Morphine glucuronide
Nadolol
Naloxone
Naltrexol
Naltrexone
Naproxen
Nefazodone
Nicotine
Nitrazepam
Nitrofurantoin
Nomefensine
Norclozapine
Norcocaine
Norcodeine
Norfentanyl
Norfluoxetine
Norketamine
Normeperidine
Normethadone
Normorphine
Noroxycodone
Noroxymorphone
Norpropoxyphene
Nortriptyline
Olanzapine
Orphenadrine
Oxazepam
Oxbutynin
Oxcarbazepine
Oxcarbazepine metabolite
Oxprenolol
Oxycodone
Oxymorphone
Oxyphenbutazone
Papaverine
Paraquat
Paroxetine
Pemoline
Penicillamine
Pentazocine
Pentobarbital
Pentoxyfylline
Pericyazine
Perphenazine
Phenacetin
Phencyclidine
Phendimetrazine
Phenelzine
Phenformin
Pheniramine
Phenobarbital
Phentermine
Phenylbutazone
Phenylephrine
Phenylpropanolamine
Phenytoin
Pholcodine
Picloram
Pimozide
Pindolol
Pipotiazine
Piroxicam
Pizotifen
PMA
Prazepam
Prazosin
Prednisolone
Prednisone
Prilocaine
Primidone
Procainamide
Procaine
Prochlorperazine
Procyclidine
Promethazine
Propafenone
Propoxyphene
Propranolol
Protriptyline
Pseudoephedrine
Psilocin
Pyrilamine
Pyrithyldione
Quetiapine
Quinidine
Quinine
Ramipril
Ranitidine
Reboxetine
Remifentanil
Risperidone
Ritalinic acid
Rivastigmine
Salbutamol
Salicylate
Scopolamine
Secobarbital
Serotonin
Sertraline
Sibutramine
Sildenafil
Sotalol
Stanozolol
Strychnine
Sufentanil
Sulfadiazine
Sulfamethoxazole
Sulfasalazine
Sulfinpyrazone
Sulfisoxazole
Sulindac
Sulpiride
Sumatriptan
Temazepam
Terbutaline
Testosterone
Tetrabenazine
Tetracycline
Tetrahydrocannabinol
Theophylline
Thiethylperazine
Thiopental
Thioproperazine
Thioridazine
Thiothixene
Tiaprofenic acid
Timolol
Tolazamide
Tolbutamide
Topiramate
Tramadol
Tranylcypromine
Trazodone
Triamcinolone
Triamterene
Triazolam
Trifluoperazine
Trihexyphenidyl
Trimeprazine
Trimethoprim
Trimipramine
Tripelennamine
Venlafaxine
Verapamil
Vigabatrin
Warfarin
Yohimbine
Zaleplon
Zanamivir
Ziprasidone
Zolpidem
Zomepirac
Zopiclone
Zuclopenthixol

In this article, the Experimental section is divided into subsections about the formation of the library, the application of the library to samples from two different labs around the world (Riverview Hospital and Athens Doping Lab), and the implementation of identifying compounds and reporting results in an automated fashion for a sample from a third lab (St. Olav's Hospital).

Experimental

Part 1 - Creating the Library

The library used in this work includes the compounds listed in Table 1, which have been analyzed in the lab of Riverview Hospital (Coquitlam, BC, Canada) using HPLC and an LC/MSD Trap XCT mass spectrometer. The library is simply called "Drugs_lib." Liquid chromatography is useful for removing background interferences from impurities that may exist even in compound standards. However, infusion using a syringe pump is also possible, especially if the standard solution is pure.

Each compound is run chromatographically as a standard with a fast elution time of about one minute. The details of the chromatography vary a little depending upon the sample, so they are not presented here. In addition, some compounds give appreciably better spectral signal in negative electrospray ionization (ESI) mode, while the analysis of some others results in much better signal using atmospheric pressure chemical ionization (APCI), whether positive or negative. However, the majority of the compounds show the best spectral response in positive ESI, especially those that are later confirmed in the analyses of St. Olav's Hospital and Athens Doping Lab.

The most relevant acquisition settings for generating the library spectra are outlined in Table 2. An Agilent LC/MSD Trap XCT mass spectrometer is used to acquire the spectra, which consist of one MS/MS (MS^2) and at least one MS/MS/MS (MS^3) spectrum. The spectra that are specific to each transition are then averaged together over the width of the eluting compound peak to produce clean spectra for each transition. The spectra are then transferred to the library for each compound so that the corresponding library entry contains one MS^2 and one or more MS^3 spectra (Figure 2).

Table 2. Mass Spectrometer Acquisition Settings for Library Creation

Parameter	Value
Ramped collision energy	0.3–2.0 V
Precursor isolation width	4 amu
Spectra acquired	1 MS, 1 MS^2, and ≥ 1 MS^3, if possible

A few comments should be made about which spectra are used in the library. First of all, although a full-scan MS, or just MS, acquisition is made as part of deciding which precursor ion to isolate and fragment, the spectrum itself is not used in the library. The reason for this is twofold. First of all, an MS spectrum may contain co-eluting interferences that degrade the spectrum, making it difficult for use in identifying unknowns.

The identification algorithm used in the Agilent ion trap software for matching each spectrum of an unknown compound with one in the library is NIST-based. Therefore, scores for Fit (F), Reverse Fit (RFit), and Purity (P) are obtained for each spectrum match. The Fit score is directly related to the number of spectral peaks in the library compound spectrum that are also found in the unknown. The converse is true for the RFit score, which is related to the number of spectral peaks in the unknown spectrum that are also in the library spectrum.

Therefore, if the library spectrum contains more peaks than the unknown, then the Fit score will be low. This is likely in the MS spectrum if there are co-eluting interferences in the spectral acquisition for the library. If the unknown spectrum contains interferences, then the RFit score will be low. As a result, it is preferable to simply not consider the MS spectra for purposes of identification.

Secondly, the most important information in the MS spectrum, which is the precursor ion mass, is stored in the MS^2 and MS^3 spectra because they are all generated from a fragmentation pathway that begins with the precursor ion.

As shown in Figure 2, each compound entry includes an MS^2 spectrum, and usually at least one MS^3 spectrum, the CAS number, chemical formula, and an image of the structure. In addition, the mass list of each spectrum undergoes a filtering in which any m/z value with an abundance less than 10 is removed from the spectrum. The reasoning for this is that the largest m/z is normalized to 999 and an abundance of 10 represents about 1.0%. The 1.0% threshold corresponds to the possible residual signal following the isolation step. That

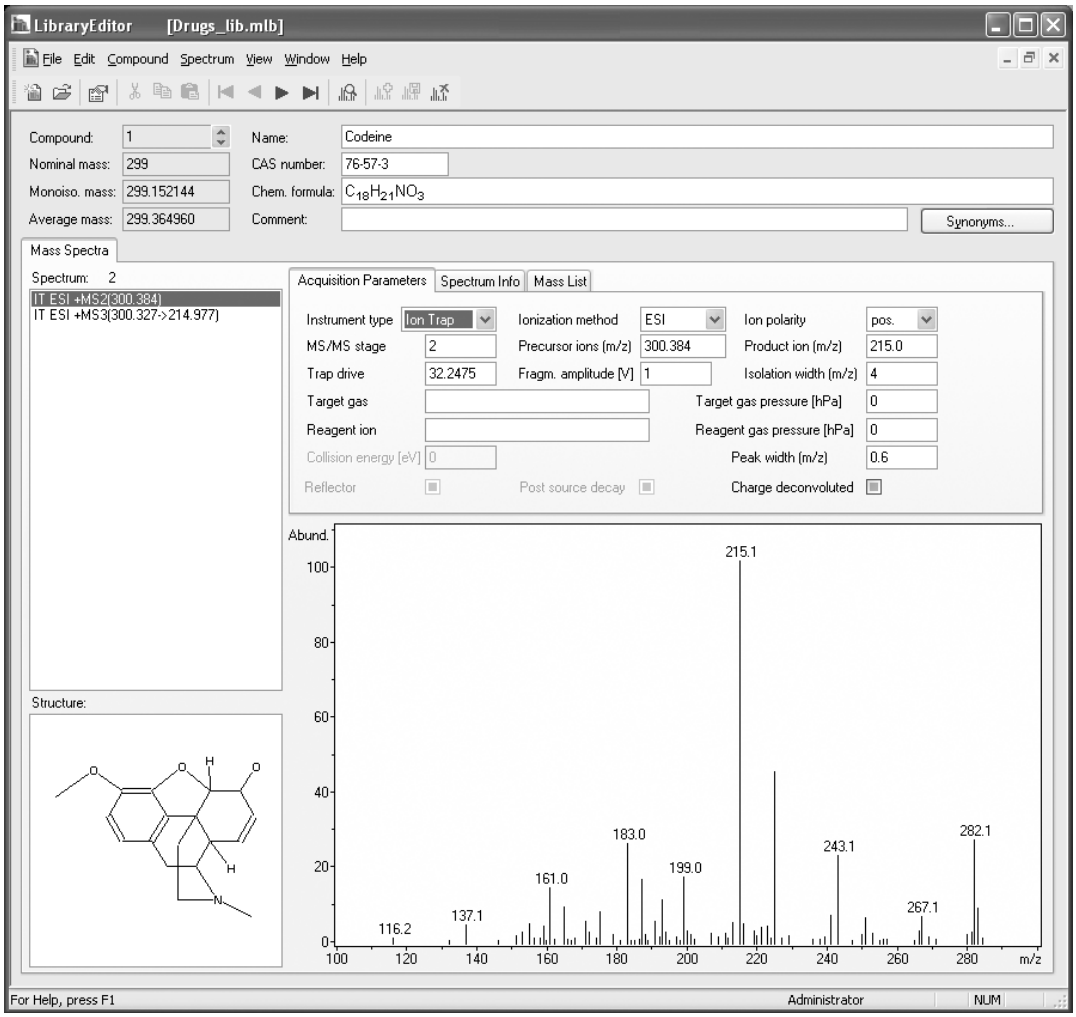

Figure 2. Library entry for cocaine compound containing one MS^2 and one MS^3 spectrum.

is, isolation of the precursor ion in an ion trap is better than 99%, but not quite 100%. Therefore, following the precursor ion isolation there may be as much as 1% of the signal remaining from an *m/z* ion outside the isolation window. Since this residual does not represent fragmentation of the isolated precursor ion, it is removed from the library spectrum.

Part 2 - Using the Library for Identification

Sample 1 (Riverview Hospital)

The first example of using this library to identify or confirm the presence of drug compounds is in a patient blood extract sample at the Riverview Hospital. Although the library spectra in Drugs_lib are obtained from standards run on the same LC/MSD Trap XCT instrument in the Toxicology Lab, the patient sample includes a blood extract matrix, which can interfere with the analyte signal as it co-elutes with the compounds of interest. Therefore, this first test of the library demonstrates the power of MS^2 to selectively remove sample matrix interferences.

The analysis of the patient sample is carried out using an "AutoMS(3)"(Figure 10) acquisition in which the signal is monitored in the MS only mode, or full- scan MS. When the intensity of any ion within the allowed selection window exceeds a minimum threshold value, that ion is then selected as a precursor ion and fragmented. Up to two precursor ions in each MS spectrum may be selected. Once the precursor ion is fragmented, any product ions exceeding a threshold for MS^3 are then fragmented. Only one precursor ion from each MS^2 spectrum may be chosen.

Since the resulting data file consists of MS, MS^2, and MS^3 spectra, a special algorithm in the software is used to generate corresponding

compounds, each of which include MS, MS^2, and MS^3 spectra related to each other by a common MS precursor ion within a time window of one minute. The time window is important because if another compound of the same *m/z* precursor ion mass in MS elutes later, it will not be confused with the earlier eluting compound.

Figure 3 shows the result of applying the special algorithm, also known as Find – Compounds | AutoMS(n), to a patient sample data file. Based on user-defined parameter settings in the algorithm, 10 compounds are found, each containing at least MS and MS^2 spectra, some containing MS^3, as shown in the figure. The next step is to identify these compounds using the Drugs_lib library, which also contains MS^2 spectra, and for most of the compounds, MS^3 spectra as well.

At this point, it should be noted that the "Identify" algorithm of the MSD Trap software is set to ignore the MS spectra of the compounds in the sample data file. Therefore, only MS^2 and MS^3 spectra of the sample will be compared with MS^2 and MS^3 spectra of the library.

The MS spectra of the sample data file are ignored for two reasons. First, the MS spectra contain co-eluting interferences, which can reduce the NIST-based scores for matching spectra. Second, the only information of importance in the MS spectrum is the precursor ion mass, which is stored in the MS^2 and MS^3 spectra anyway. If it is of interest to use adducts like sodium or potassium for identification, the MS data is still there for further investigation later.

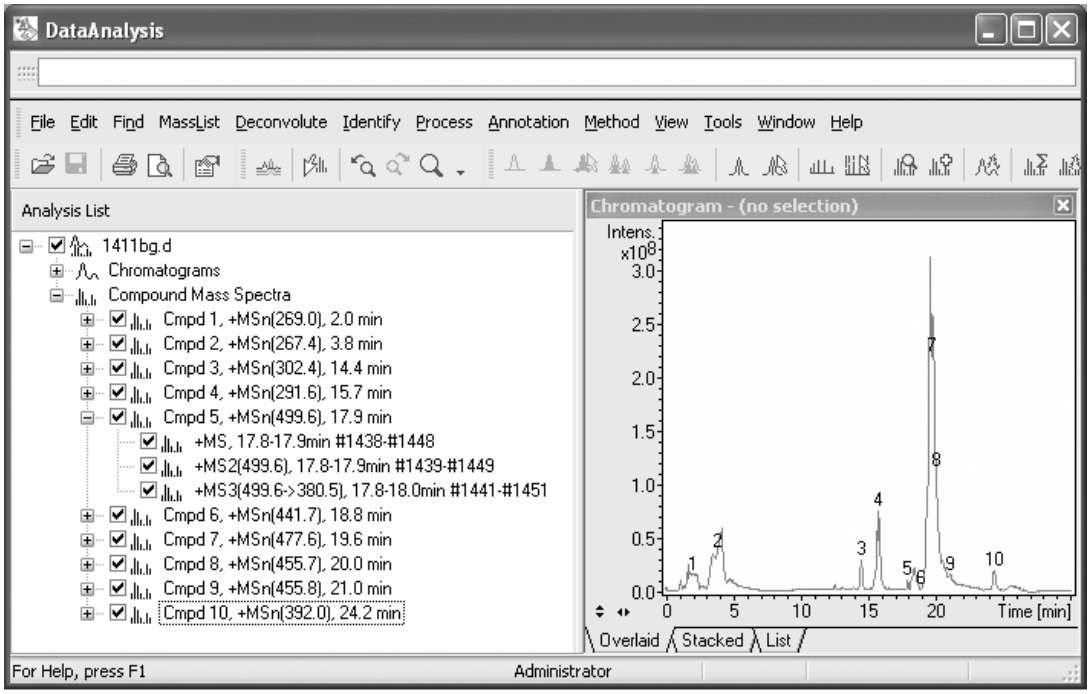

Figure 3. Ten compounds found in the patient blood extract sample data file named "1411bg." Note that the compounds found are based on user-defined parameter settings.

After identifying the compounds, any of the compounds that are not identified can be removed from the analysis list by using the Visual Basic script shown in Figure 4. By implementing this script, the number of relevant compounds is reduced from 10 to 5, as seen in Figure 5.

The five compounds are identified as shown in Figure 6. Obviously, compounds 4 and 5 are the same compound and representative of a large peak with substantial intensity spread out over more than one minute in elution.

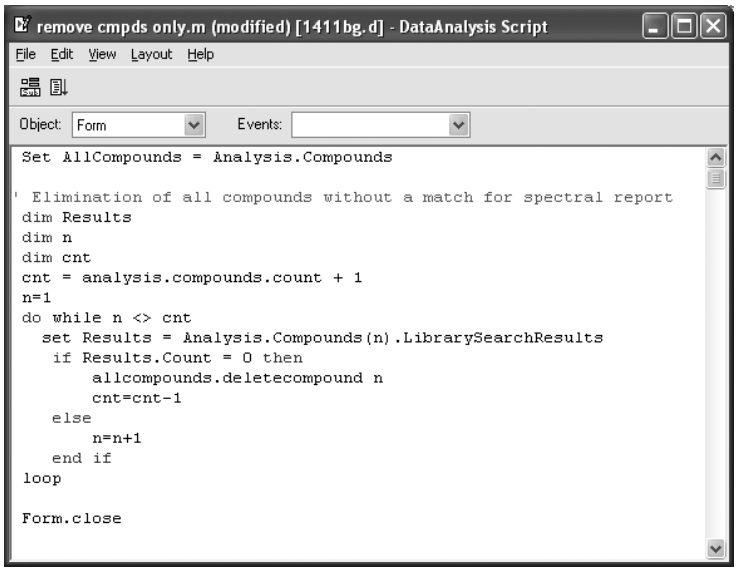

Figure 4. Visual Basic script used to remove any compounds not identified by the library. This can make reporting results more simplified.

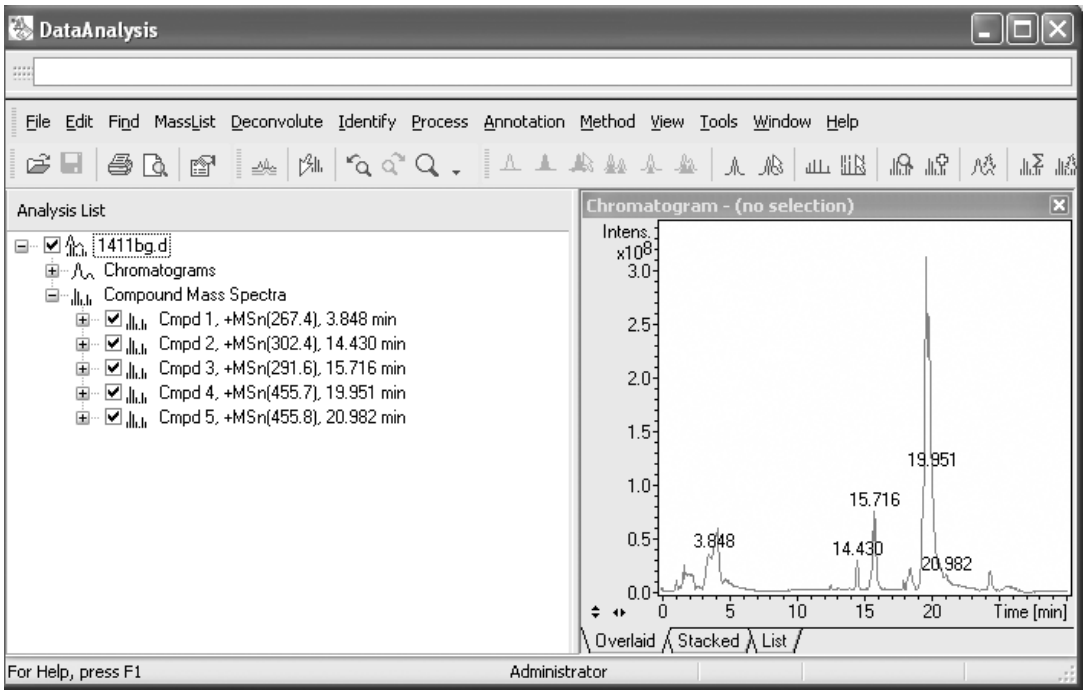

Figure 5. After searching the compound spectra of the sample data file, only five are identified with the library. The script shown in Figure 4 removes the compounds not identified.

Library Search Report - AutoMS(n)

Compound List:

#	RT [min]	MS(n) Isol. m/z	Compound Name	Fit'	RFit'	Purity'
1	3.848	267.4	Atenolol	998	998	998
2	14.430	302.4	Temazepam	998	998	998
3	15.716	291.6	Azatadine	1000	997	997
4	19.951	455.7	Verapamil	998	998	998
5	20.982	455.8	Verapamil	998	998	998

Figure 6. Compounds identified in patient blood extract sample include atenolol, temazepam, azatadine and verapamil.

The scores shown in Figure 6 (Fit', RFit', and Purity') are effective scores that take into account the matching upon multiple spectra between the samples' compounds and those in the library at both the MS^2 and MS^3 levels. As an example, consider the Fit' score for atenolol. The equation used for determining any effective score is given below.

$$\text{Score'} = \left(\frac{\sum_{i=1}^{M} \text{Score} \times \text{Match}}{M \times 10^6} \right)^{1/N} \times 1000$$

Score' represents the effective score while Score represents the individual scores for each matched spectrum in the data file. The value for N is the total number of spectra and M is the number of identified spectra.

The Match score has to do with user-defined matching parameters. Looking at the results for atenolol in the data file (see Figure 7) the individual Fit scores for the identified spectra of Compound 1 are 991 for MS^2 and 1,000 for MS^3. The match values are maxima of 1,000 each because an MS^2 spectrum of the library has been matched to the MS^2 spectrum of Compound 1 and likewise for the MS^3 spectra. Had an MS^3 spectrum been matched to an MS^2 spectrum, the Match value would be 500.

The effective score for Fit, or Fit', is therefore $[(991 \times 1000 + 1000 \times 1000)/(2 \times 10^6)]^{1/2} \times 1000 = 998$, which is shown in Figure 6.

While the Fit, RFit, and Purity scores for each spectrum are from NIST-based algorithms, the handling of multiple spectral matches and different levels of MSn is unique to the MSD Trap data analysis software. This is an intelligent way of deriving an overall score, or effective score, for the compound in question.

Taking a look at the actual spectral matches, atenolol is once again used as an example. Figure 8 shows the MS^2 and MS^3 matches as seen in the remainder of the library report shown in Figure 6.

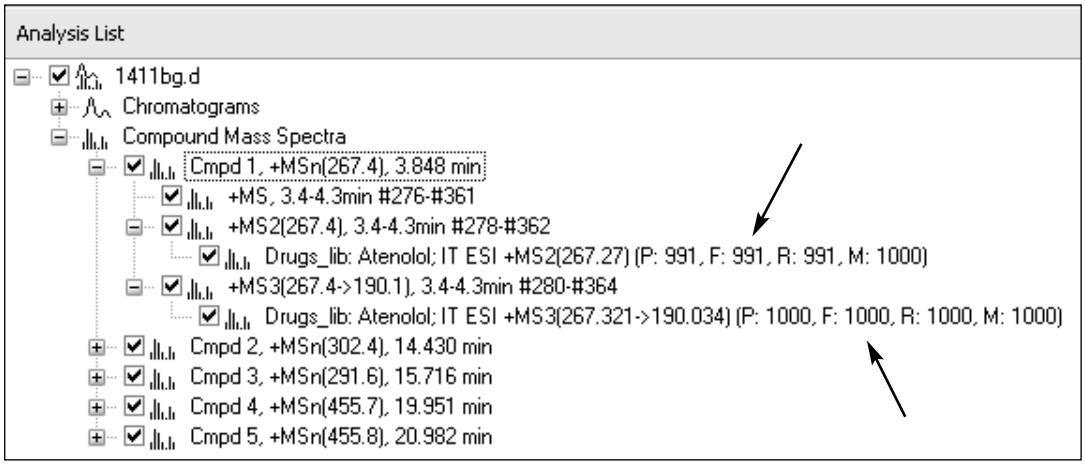

Figure 7. Individual library search scores shown for Compound 1 ("Cmpd 1"). The Fit score for the MS^2 spectrum is 991 and the Fit score for the MS^3 spectrum is 1,000, the maximum possible.

Figure 6 also shows that Compound 1 was only identified at atenolol and no other drug in the library. This demonstrates the power of using both MS^2 and MS^3 spectra for identification as well as the reproducible nature of using ramped collision energy to form fragments across a wide mass range.

Sample 2 (Athens Doping Lab)

The presence of corticosteroids in a urine extract sample is both confirmed and quantitated through targeted MS^2 with ramped collision energy, or SmartFrag. While it is better to use a specific collision energy for quantitation, the implementation of SmartFrag can still give good sensitivity in full-scan MS^2 while at the same time providing the spectra for identification.

In a particular sample known as "CMX_12," the presence of triamcinolone, corticosterone, hydrocortisone, prednisolone, prednisone, methylprednisolone, and dexamethasone is confirmed through library identification and quantitated. Figures 9A and 9B show two examples. Although no MS^3 data is acquired for this sample, the scores for the MS^2 matches are all greater than 900. However, it should be noted that the effective scores would be much closer to the maximum of 1,000 if MS^3 spectra are also acquired. The reason it is not included here is to save time for acquiring enough signal in MS^2 for quantitation.

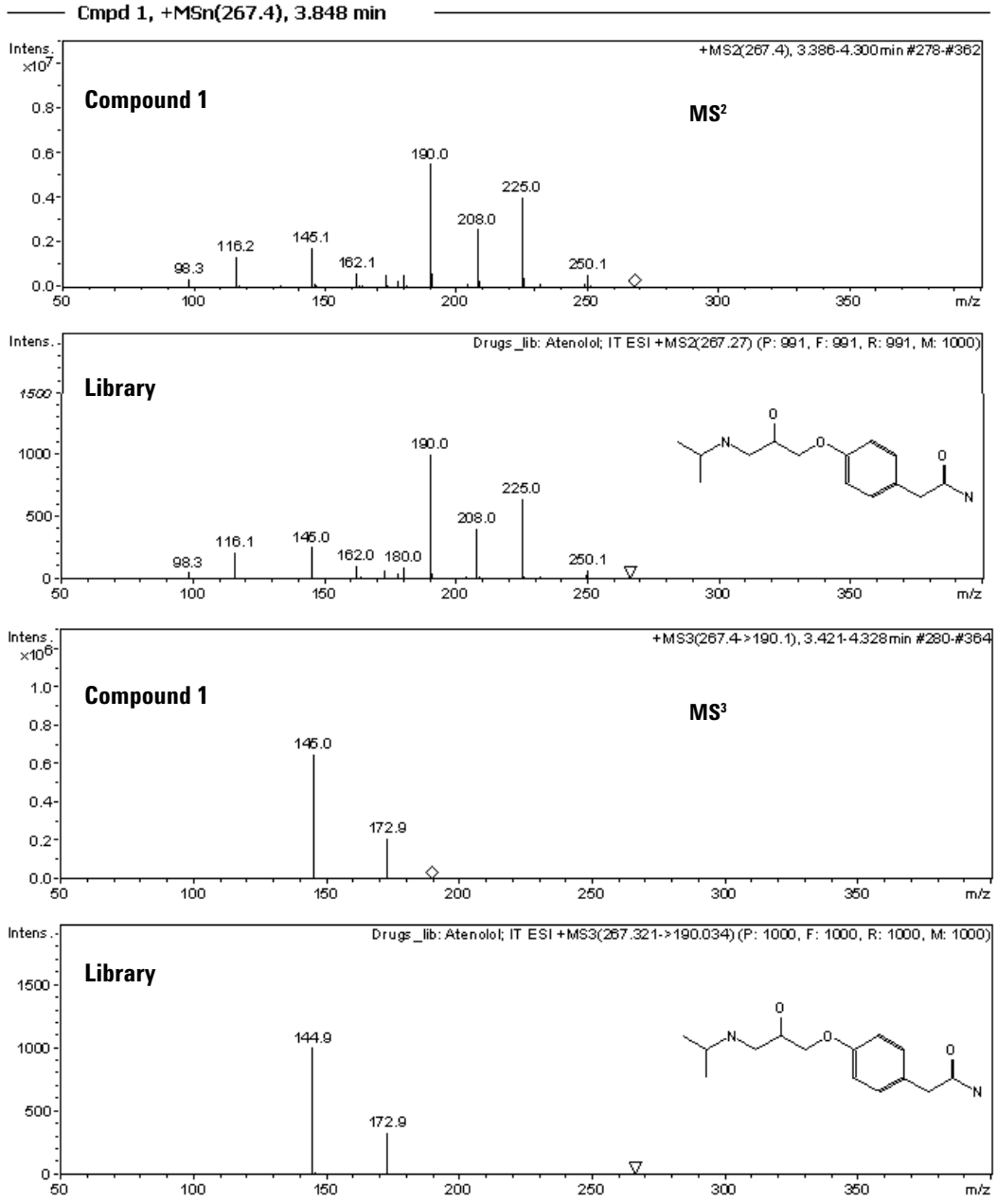

Figure 8. Matching of Compound 1 spectra to library spectra of atenolol in Drugs_lib.

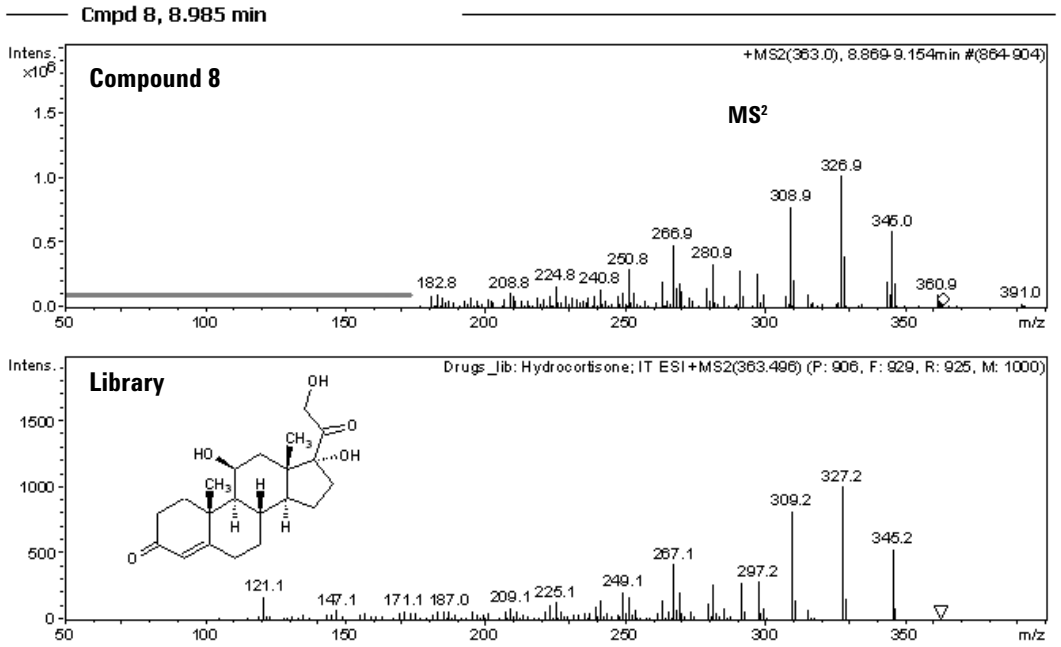

Figure 9A. Matching of Compound 8 spectra to library spectra of hydrocortisone in Drugs_lib.

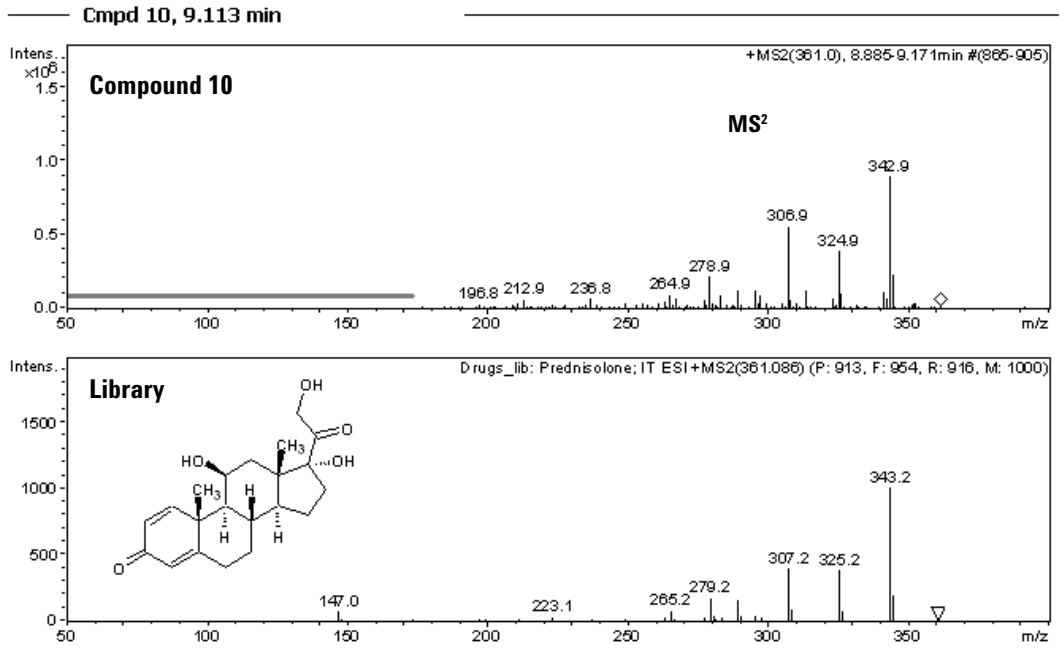

Figure 9B. Matching of Compound 10 spectra to library spectra of prednisolone in Drugs_lib.

Because there are no MS^3 spectra to also search, the effective scores reported in the library report, using the equation given above, are the same as those shown in Figures 9A and 9B. Furthermore, it should be noted that even though the mass assignments differ by as much as 0.3 amu, the spectra are remarkably similar in appearance for comparing data from an Agilent ion trap in the lab of Riverview Hospital and an Agilent ion trap in the Athens Doping Lab. In fact, the two models of ion traps are different as well. The ion trap of the Athens Doping Lab is the LC/MSD Trap SL and predecessor to the LC/MSD Trap XCT, which is at Riverview Hospital.

Part 3 – Using the Library for Automated Screening

High-throughput screening of drugs of abuse is performed in the Department of Clinical Pharmacology (DCP) at St. Olav's Hospital

(Trondheim, Norway) by LC/MS, which performs more than one million analyses per year. Traditionally, screening is performed by immunoassay, which can be expensive and less selective than mass spectrometry. The samples that are positive in the screening process are then traditionally confirmed using GC/MS, which requires derivatization as part of the sample preparation process. In such an environment, confirming the presence of compounds using an LC/MS/MS library in an automated fashion can be extremely valuable.

In this part, the results of carrying out a confirmation analysis on an Agilent LC/MSD Trap XCT mass spectrometer, located at DCP, is demonstrated. Once again, SmartFrag is used. This time, the compounds of the screened samples are analyzed by the LC/ion trap using data-dependent acquisition in which a list of precursor ions is referenced. Therefore, AutoMS(3) is once again used, but this time there is an inclusion list of *m/z* values corresponding to the presence of molecular ions being confirmed. In this way, the ion trap is monitoring the MS acquisition to see if any of these ions appear above a particular background threshold. If they do, the ion trap will isolate and fragment them. If any product ions in the MS^2 stage are above a user-defined level, then the corresponding product ions will in turn be isolated and fragmented.

The acquisition settings are shown in Figure 10. The list of ions in the Include list shown in Figure 10 is defined in Table 3.

Once data acquisition is completed for each data file in a sequence, the data file is automatically sent to the LC/MSD Trap DataAnalysis program, where a Visual Basic script is applied to first find all compounds in the data file. This is done as discussed previously (see also Figure 3) by generating all possible MS, MS^2, and MS^3 extracted ion chromatograms (EICs), locating the peaks in these EICs and then associating the spectra under these peaks with each other according to the MS-based precursor ion *m/z* from which they came. Each grouping of spectra is known as a compound.

The compound MS^2 and MS^3 spectra are then automatically searched against the Drugs_lib library database, and NIST-based scores for Fit, RFit, and Purity are determined for each spectrum relative to a library spectrum. To take advantage of the LC/MSD Trap preserving specific pathway

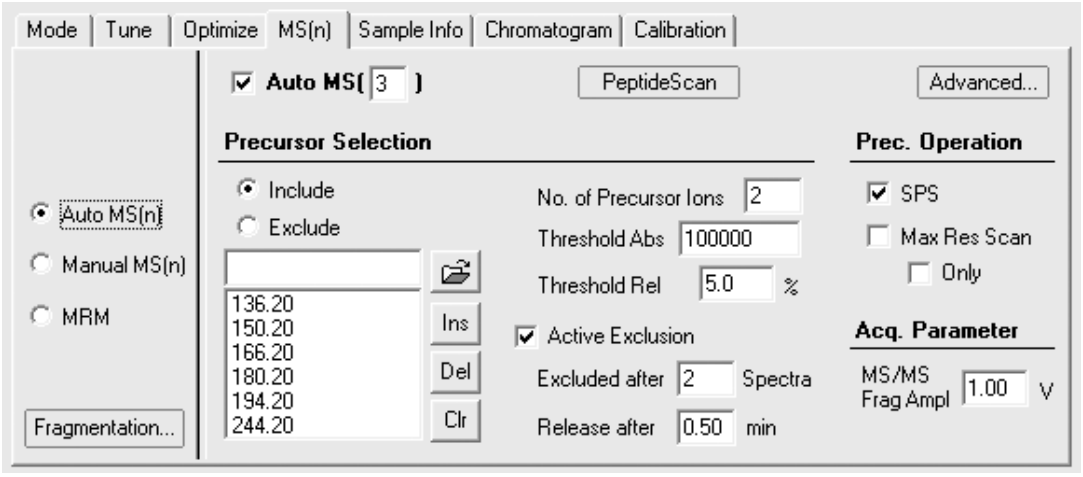

Figure 10. AutoMS(3) settings for confirmation acquisition.

Table 3. List of Precursor Ions Used for Data-Dependent MS^2 and MS^3

Precursor (*m/z*)	Compound	Precursor (*m/z*)	Compound
136.2	Amphetamine	286.2	Morphine
150.2	Methamphetamine	300.2	Codeine
166.2	Ephedrine	310.0	Methadone
180.2	MDA	314.2	Ethylmorphine
194.2	MDMA	316.2	Oxycodone
244.2	Phencyclidine	399.2	Pholcodine

information at the MS^2 level, only the MS^2 spectra of the library are searched for comparison with the MS^2 of the sample compound.

At the MS^3 level many of the product ions are the same, even though different pathways through different MS^2 product ions are followed. Therefore, the search algorithm allows for the comparison of sample compound MS^3 spectra with MS^3 library spectra where the MS^2 product ions may differ. Nevertheless, all searches are limited to comparing the same MS^2 transitions between the sample compound spectra and the library spectra, as well as comparing the corresponding MS^3 spectra, even though the MS^2 product ions may differ.

Finally, those compounds not identified are left out of the report by removing them using the automation script, as was shown in Figure 4.

A nice example of using the ion trap library is shown in the automatically generated library report of Figures 11 and 12 for a data file called "012-1901." The sample involved has been screened by an Agilent LC/MSD single quadrupole mass spectrometer, and the presence of amphetamine, methamphetamine and codeine has been detected. The codeine is quantified at a level of 6.5 µg/mL of urine, but the signal for the other two drugs is too low to measure with confidence.

The results in the report demonstrate not only the power to identify the compounds expected in the sample, but also the sensitivity to detect two other compounds not seen in the screening step: morphine and MDMA. The scores shown in the report are effective scores based on the mathematical consideration of both the MS^2 and MS^3 level matches.

Figure 12 shows good spectral matches for codeine between the sample analyzed in the St. Olav's lab and the library developed in the Riverview Hospital lab. The importance of using MS^3 spectra is demonstrated by the fact that the individual scores for the match at the MS^2 level are all just under 900, but the scores at the MS^3 level are close to 1000, so that the overall effective scores shown in Figure 11 are well over 900.

Library Search Report - AutoMS(n)

St. Olav's Hospital
Department of Clinical Pharmacology
7006 Trondheim, Norway

Analysis Name: 012-1901.D **Instrument:** LC-MSD-Trap-XCT **Print Date:** 10/12/200612:24:42 AM
Method: BAS_SB.M **Operator:** Administrator **Acq. Date:** 8/28/2006 6:02:18 PM
Sample Name: 109647

Compound List:

#	RT [min]	MS(n) Isol. m/z	Compound Name	Fit'	RFit'	Purity'
1	2.0	286.6	Morphine	975	993	971
2	2.1	194.4	MDMA	978	954	934
3	2.8	300.4	Codeine	989	990	989
4	2.8	136.0	Amphetamine	1000	1000	999
5	3.6	150.4	Methamphetamine	998	912	910

Figure 11. First page of report listing compounds identified.

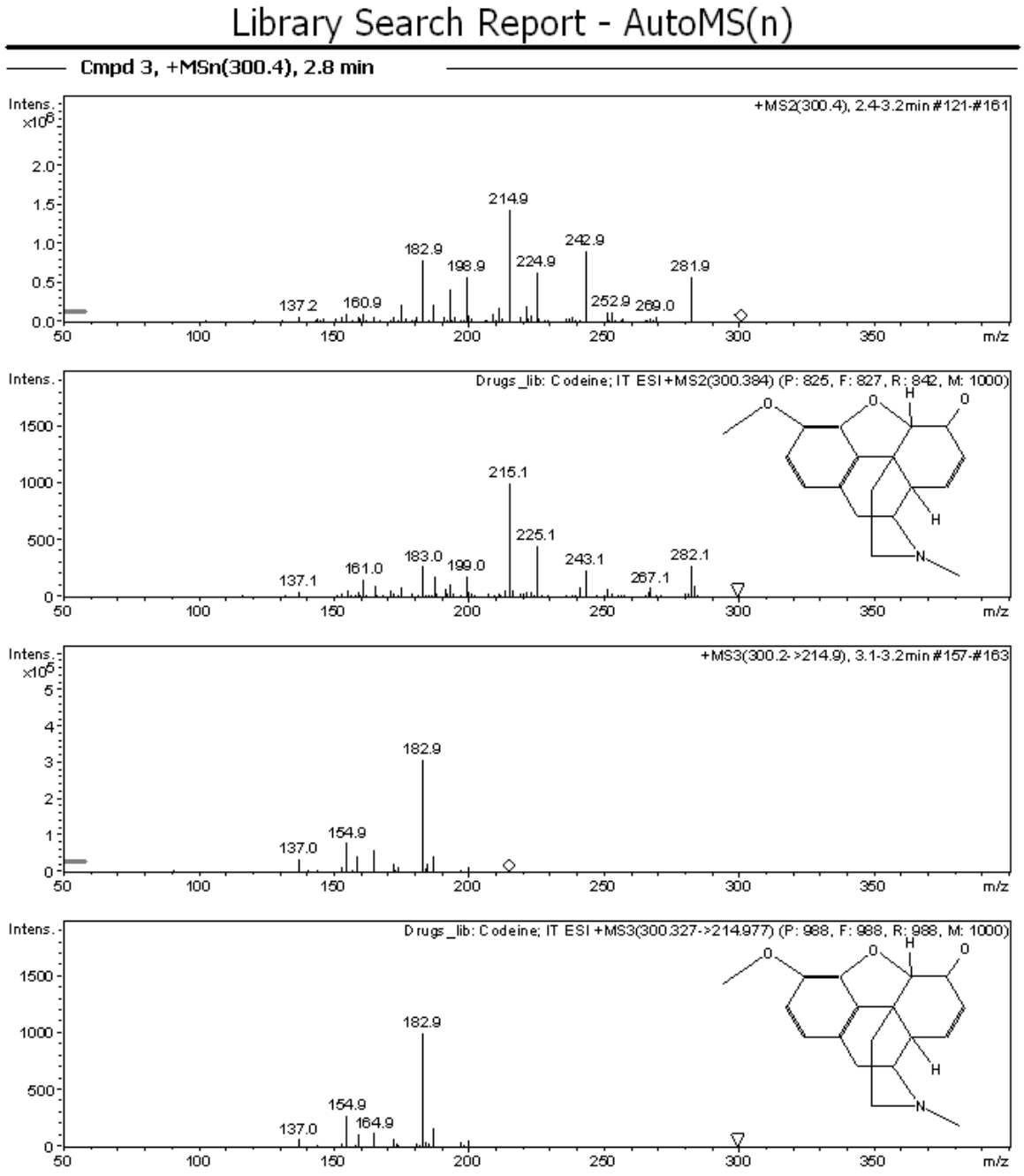

Figure 12. A page in a report showing spectral matching.

Conclusions

MS^2 and MS^3 spectra, acquired using ramped collision energy, is an effective technique for confirming the presence of compounds in a sample. MS^3 spectra increase the specificity of analysis and an overall scoring technique needs to account for matching spectra at both the MS^2 and MS^3 levels. Calculating effective scores from the individual NIST-based scores for each spectrum takes into account the overall identification of the compound of interest. To increase productivity, the data processing, compound identification, and reporting must be automated.

For More Information

For more details concerning this technical overview, please contact Michael Zumwalt at Agilent Technologies, Inc.

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